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Although most of the tumors associated with neurofibromatosis type 1 (NF1) are benign in nature, malignant transformation of a subset of NF1 tumors is a serious complication, often leading to the death of the patient. This is true for NF1-associated juvenile chronic myelogenous leukemia (JCML), known to progress into acute leukemia. Our goal is to investigate the events which lead to the transformation and leukemic progression of NF1-associated JCML using a mouse model that we have developed. This model system takes advantage of transgenic mice that harbor one mutant allele of the *Nf1* gene, but require further mutations for transformation. This past year, we have backcrossed this mutant *Nf1* allele for three generations to a strain of mouse that expresses a murine leukemia virus (MuLV). In this system, the MuLV acts as a mutagen to activate cooperating cellular proto-oncogenes or inactivate tumor suppressor genes, resulting in accelerated tumor development. So far, we have been able to generate 17 new tumors to add to our panel and have many other animals in aging. Furthermore, we have initiated experiments directed at cloning the affected genes based on their proximity to the newly integrated proviral DNA.

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Carrie Brannan 10/1/98

PI - Signature Date

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## Introduction

Children with neurofibromatosis type 1 (NF1) have a markedly increased risk for juvenile chronic myelogenous leukemia (JCML) and monosomy 7 (Mo7) syndrome. JCML, a myelodysplastic syndrome, and Mo7 syndrome, a myeloproliferative disorder, share many features including similar age of onset, a tendency to affect boys, prominent enlargement of the liver and spleen, leukocytosis, and the absence of the Philadelphia chromosome. Both JCML and Mo7 syndrome have a poor prognosis, with either progression to acute myeloid leukemia (AML) after a short period, or death from incurrent problems (1). It has been estimated that approximately 10% of children with myeloid leukemia also have NF1 (1-4). This frequency is likely to be higher, however, as the peak years of incidence of childhood leukemia occurs at an age when NF1 in children often goes undiagnosed (5).

In 1994, Shannon *et al.* published their finding that 5 out of 11 bone marrow samples obtained from children NF1 and JCML or Mo7 showed loss of heterozygosity (LOH) for markers within and near the *NF1* locus. In each case, the remaining *NF1* allele was inherited from the parent with NF1, indicating that the normal allele was deleted (6). These results are consistent with the hypothesis that *NF1* loss predisposes myeloid cells to leukemic transformation in children. Further, this group has found that while Ras point mutations are often found in JCML patients without NF1, they are not found in JCML patients with NF1 (7), suggesting that *NF1* gene loss and activating Ras point mutations are functionally equivalent. This hypothesis is supported by the fact that neurofibromin, the protein product of *NF1*, contains a region that has extensive homology with the catalytic domain of GTPase activating proteins (GAPs) that are known to accelerate the intrinsic GTPase activity of Ras, thereby negatively regulating Ras-GTP levels (8). While these data indicate that the loss of the *NF1* gene product is an important step in the pathogenesis of myeloid leukemia in children with NF1, it is not possible to determine from these data which specific disease symptoms are associated with this event.

To investigate the phenotypic consequence of the loss of *NF1*, we reasoned that a homogeneous genetic background would be extremely helpful. Therefore, we developed a mouse model for human NF1-associated JCML (9). This model involves takes advantage of hematopoietic cells derived from an *Nf1* knock-out line of mice, $Nf1^{Fcr}$, that we have previously created (10). Since this mutation was lethal in the homozygous state between embryonic day 13.5 to 14.5, we obtained *Nf1*-deficient hematopoietic stem cells by intercrossing two heterozygous $Nf1^{Fcr}$ mice and sacrificing pregnant females at 13.5 days of gestation. At this time in development, the fetal liver is the primary site of

hematopoiesis and is known to contain hematopoietic stem cells capable of long term reconstitution upon transplantation into lethally irradiated mice (11). Therefore, we isolated livers from the embryos for use in a series of *in vitro* and *in vivo* experiments.

Initially, fetal livers of homozygous, heterozygous and wild-type embryos were analyzed *in vitro* by cytokine-induced colony formation in methylcellulose and we found:

**(1)** Although there were equal numbers of colony-forming units in all three genotypes, a subset of the colonies in the homozygous $Nf1^{Fcr}$ mutant cultures were larger. These larger colonies contained developing neutrophils and/or monocytes, two of the primary cell types affected in JCML.

**(2)** Homozygous mutant hematopoietic stem cells were hypersensitive to the cytokine GM-CSF *in vitro*. This mimics what is seen with leukemic cells from JCML patients that have been reported to be hypersensitive to GM-CSF (12).

**(3)** Lack of the *Nf1* gene resulted in an increased and prolonged rise in Ras-GTP levels in myeloid cells following GM-CSF stimulation *in vitro* . As the GM-CSF receptor is known to function, in part, through the Ras pathway (13), this data supports the hypothesis that the observed GM-CSF hypersensitivity was due to the deregulation of Ras caused by the absence of the neurofibromin.

Next, fetal livers of homozygous, heterozygous and wild-type embryos were analyzed *in vivo* by transplantation into lethally irradiated mice. Using these assays, we found:

**(4)** Mice reconstituted with homozygous mutant cells developed a chronic myeloproliferation syndrome that was characterized by leukocytosis with high peripheral blood neutrophil and monocyte counts, often with circulating immature elements. All the mice reconstituted with control cells had normal white blood cell levels.

**(5)** Within the first 1.5 years post reconstitution, 27% of the mice reconstituted with homozygous mutant cells died from disease progression while the remaining 73% of genetically identical mice remained viable. This strongly suggests that while loss of *Nf1* is sufficient to cause chronic myeloid leukemia, hypersensitivity to GM-CSF, and elevated levels of Ras-GTP levels, it is not sufficient to cause acute leukemia. This data indicates that additional somatic mutation is required for progression to more acute disease.

The goal of this proposal is to identify these additional somatic mutational events. We will use a powerful genetic system that we have developed to identify regions, and eventually genes, that when mutated, are responsible for the fatal progression of NF1-associated JCML. Our strategy is to breed the mutant *Nf1* allele, $Nf1^{Fcr}$, onto a strain of

mice that expresses murine leukemia virus (MuLV) and exhibits a high incidence of acute myeloid leukemia. In this system, the MuLV acts as a somatic mutagen to activate cooperating cellular proto-oncogenes or inactivate tumor suppressor genes, resulting in accelerated tumor development. Since MuLVs activate proto-oncogenes by integrating nearby or inactivate tumor suppressor genes by integrating within the gene, the affected genes can thus be identified and cloned using these somatically acquired viruses as signposts. The most promising loci are the ones that have sustained proviral insertion in tumors of multiple mice. The identification of a so-called "common site of viral integration" strongly indicates that the region harbors a gene, that when mutated by the virus, is directly involved in the development of myeloid leukemia. Preliminary studies using this system have demonstrated that:
***(1)*** Tumor progression is accelerated in these heterozygous *Nf1* mutant mice relative to wild-type controls.
***(2)*** Tumor tissue isolated from the majority of these heterozygous mice lack the normal *Nf1* allele due to either loss of heterozygosity or to viral insertion.
***(3)*** Each of these tumors contain at least one somatically acquired provirus which can serve as a tag to identify genes involved in *Nf*1-dependent tumor progression.
***(4)*** Finally, after isolating a site viral integration from one tumor, we determined that this site is similarly affected in two other independently-derived tumors. This strongly indicates that this region harbors a gene that is involved in tumor progression.

**Technical Objective 1: identify regions of the genome that cooperate with loss of *Nf1* in myeloid leukemia to cause tumor progression.** We propose to create a large panel of murine acute myeloid tumors that lack *Nf1* expression. This panel will be used to identify additional common sites of viral integration.

**Technical Objective 2: characterize a locus containing a gene involved in tumor progression.** Having already identified one common site, we propose to identify and characterize the affected gene.

## Body of the Progress Report

We will review our progress during the first 12 months of this grant in relation to each of the tasks outlined in our Statement of Work.

**Technical Objective 1: Identify regions of the genome that cooperate with loss of *Nf1* in myeloid leukemia to cause tumor progression.** Our objective is to produce a panel of approximately 100 tumors derived from heterozygous N3 generation BXH-2 *Nf1*$^{Fcr}$/+ mice. This panel will then be used to identify new common sites of viral integration. We have divided this objective into six tasks. We will report our progress on the first four tasks.

**Progress on Task 1:** Months 1-10: Produce the F1, then the N2, followed by the N3 generation of BXH-2 *Nf1*$^{Fcr}$/+ mice.

Our initial strategy was to complete production of each generation before going onto the next. However, in practice, this strategy has not worked, mainly because the parental BXH-2 strain to which we are backcrossing has had small litters. Therefore, we have staggered the breeding to keep in line with the BXH-2 production. This makes our life a little more complicated and has extended the time frame of this task, but the modified breeding scheme seems to be working out. At the present time we have finished all the matings to produce F1 mice, we have several N2 mating cages in service, and nine mating cages producing N3 generation mice. Therefore, we have a constant stream of N3 mice going into aging.

**Progress on Task 2:** Months 8-17: Aging 150 N3 generation of BXH-2 *Nf1*$^{Fcr}$/+ mice.

During the past year, we have had a total of 67 N3 generation of BXH-2 *Nf1*$^{Fcr}$/+ mice in aging. Of these mice, 29 have developed acute leukemia and have been sacrificed. The 38 animals that are currently in aging are being examined once per day to identify those animals that are ready to be sacrificed. Additional N3 mice will soon be added to this aging study upon weaning from the mating cages described in Task 1.

**Progress on Task 3:** Months 9-18: Collect moribund animals. Process tumor samples: assess the status of the wild-type *Nf1* allele; determine the number of somatically acquired viral integrations; phenotypic analysis.

Prior to this grant, we had generated a panel of 13 N3 BXH-2 *Nf1*$^{Fcr}$/+ tumors that we had previously shown to have lost the remaining normal *Nf1* allele, either due to loss of heterozygosity (LOH) or due to a viral insertion in the *Nf1* gene (*Evi-2*). During the past 12 months of this grant, we have generated, aged and sacrificed 29 additional mice. We have characterized tumors from 25 of these mice (the tumors of the remaining 4 have been properly stored, but are in line to be characterized). Of these 25, we have found an additional 17 that have lost the remaining *Nf1* allele. This brings the total up to 30

tumors, approximately one-third of the way toward our 100 tumor goal. For these 17 tumors, we have determined the number of the of somatically acquired viral integrations in each of the tumors and consulted with our hematopathologist, Dr. Almasri, to grade the severity of the disease. We have been pleased to find that the histopathological profile has been very consistent from tumor to tumor.

**Progress on Task 4:** Months 1- 24: Clone somatically acquired proviruses from tumor samples which harbor defects in the wild-type *Nf1* allele and have only one or two somatic proviral insertions. Prepare genomic flanking probes to screen tumor panel.

For now, we have set our sights on the analysis of five tumors that fit the criteria. We have successfully cloned multiple proviruses from individual sized-fractionated phage libraries that we have generated from each tumor. However, to our surprise, a large percentage of these proviruses do not recognize a rearrangement in the tumor from which it was cloned. This indicates that we have cloned rare proviral insertions that do not represent the viral integrations that we are after. This was unexpected as these rare insertions were only cloned occasionally in the course of the pilot experiments. Another problem has been that we have had difficulty in finding repeat free flanking probes. This, of course, has hampered our ability to use the flanking region as a probe to determine if we can detect a rearrangement in the tumor. Currently, we are sequencing flanking DNAs to identify fragments that do not contain repetitive elements. Based on sequencing, we have one promising candidate in which the virus has inserted into an area rich in the dinucleotide CpG. This is potentially very exciting as a CpG island often indicates the presence of a nearby gene.

**Task 5:** Months 18-19: Prepare Southern blot containing the panel of tumor DNA samples which harbor defects in the wild-type *Nf1* allele. Screen with candidate genes.
*Work on this task has not yet been initiated.*

**Task 6:** Months 18 - 36: Use probes that recognize common sites of viral insertion to isolate cosmid DNA. Begin studies to identify and characterize affected gene.
*Work on this task has not yet been initiated.*

**Technical Objective 2: Characterize a locus containing a gene involved in tumor progression.** Our objective is to identify the gene that is affected by a common site of viral integration. We have broken this objective into 4 tasks. Below, we have combined the progress reports for the first two of these tasks.

**Progress on Task 1:** Months 1-3: Exon trap cosmid isolated with flanking probe.
**Progress on Task 2:** Months 4 - 6: Determine if exons are candidate genes.

We had previously isolated a probe flanking the proviral integration in a tumor which we had shown lacked a normal copy of the *Nf1* gene. We showed that this flanking probe recognized a similar viral insertion in two other BXH-2 tumors, and that one of these BXH-2 tumor had sustained an additional viral integration in *Nf1*. Together, these data indicated that we had identified a locus that upon insertional mutagenesis, was capable of cooperating with the loss of *Nf1* to cause acute myeloid leukemia. Therefore, during the past year, we have sought to identify the gene affected by the proviral insertion.

We began by isolating a 40 kb mouse genomic clone from a cosmid library using the flanking probe. We exon trapped this cosmid to identify putative exons in the vicinity of the proviral insertion (14). We first shotgun cloned the cosmid DNA into the splicing vector, pSPL3. These subclones were then pooled and transfected into COS-7 cells. RNA was isolated from transiently transfected cells and subjected to RT-PCR analysis using primers complementary to the splicing vector. The PCR products were then subcloned into an ampicillin resistant vector and the colonies screened by PCR. We single track sequenced 22 clones containing PCR products larger than would be expected by splicing of the vector alone. We were able to divide these 22 clones into 9 different classes, each with multiple members, indicating that we had obtained very good representation of the trapped clones. Of these 9 clone classes, one was found to be due to cryptic splicing of the pSPL3 vector and one was found to be a mouse B1 repetitive element. The other 7 clones, were used to search both the EST and Genbank databases and none were found to be homologous to any reported sequence. This indicated that these 7 "exons" either belonged to one or more novel genes or were not transcribed at all. To determine if any of these 7 "exons" were transcribed, we used them as probes on a tissue Northern blot (polyA selected mRNA from adult kidney, lung, spleen, brain, liver, ovary, testis, as well as embryos at 13.5, 14.5, and 15.5 days of development). No signal was observed for any of the "exons".

Our next strategy was to determine which these 7 clones mapped near to the site of viral integration, and we therefore established an *Bam*HI restriction map of the cosmid. Using this map, we found that the viral integration mapped within a 2 kb *Bam*HI

fragment which itself was located less than 7 kb from one end of the cosmid. We sequenced this 2 kb *Bam*HI fragment and determined that there was no homology in the database outside of several repetitive elements. Further, this 2 kb fragment did not contain a CpG island which might have indicated the presence of a nearby gene. We then placed each of the 7 "exons" onto the *Bam*HI restriction map and found that while 5 of the clones mapped to the far side of the cosmid, 2 of these "exons" mapped closer to the site of integration: one, called 2-30, was located at the very end of the cosmid (over 7 kb away) and contained an open reading frame; and the other "exon", called 1-8, mapped to on the opposite side of the site of proviral integration, less than 4 kb away, but did not contain any open reading frames. To determine if either of these two "exons" were conserved, we probed a Zoo blot with these two sequences and found that while 1-8 recognized many bands in mouse, human, monkey, dog and chicken DNA, in contrast, 2-30 detected only a single band in mouse, monkey, dog, and chicken DNA, and 3 bands in human DNA.

The fact that 2-30 contains an open reading frame and appears to be conserved in evolution suggests that 2-30 is likely to be transcribed. To get around the problem that the "exons" might be too small to use as probes on a Northern, we isolated the 0.75 kb *Bam*HI-*Not*I end fragment containing "exon" 2-30. We used this 0.75 kb probe on a Northern blot of tissue RNA and determined that this probe recognized two bands in liver RNA, one 1.5 kb and the other 4.5 kb in size. We confirmed the expression of the 2-30 exon in liver using RT-PCR. These data demonstrate that 2-30 is transcribed. To determine if expression of this exon was altered in the tumor harboring the viral integration, we hybridized a Northern blot made with RNA isolated from BXH-2 $Nf1^{Fcr/+}$ mice tumors, including the tumor from which the viral integration was cloned. We did not detect a signal in any tumor. This result indicates that this exon is not utilized in this tumor tissue. However, it is formally possible that 2-30 belongs to a gene which exhibits tissue-specific alternative splicing. Therefore, we are currently screening a liver cDNA library to isolate additional exonic probes which we can use on the Northern blot of RNA derived from the tumor tissue.

Since we have yet to convince ourselves that we have identified the gene affected by the proviral integration, and because the site of integration maps close to the end of the cosmid, we have decided to extend our analysis beyond this 40 kb region. We therefore screened the Research Genetics BAC library and obtained one BAC clone containing over 120 kb of genomic DNA. We are currently taking two different approaches to identify genes contained on the BAC. The first approach is to exon trap the BAC as we have done with the cosmid. The second approach is to shotgun sequence the entire clone. We

have grow up BAC DNA and then randomly sheared this DNA using a nebulizer. This randomly cleaved DNA has been cloned into a plasmid and we have begun sequencing random insert containing subclones. Both of the exon trap and the sequencing approach coupled with computer analysis of the data should allow us to identify any genes located on the BAC. Once we know of all the genes, then we can begin to determine which of them has been affected by the proviral integration in the tumor.

**Task 3:** Months 7-18: Isolate cDNA clones. Determine the genomic structure and normal expression pattern of the gene. Determine orientation of the gene relative to the viral integrations. Establish if an virus activates or eliminates gene expression. *Due to the delays in identifying the affected gene encountered in Tasks 1 and 2, work on this task has been delayed.*

**Task 4:** Months 13-36: Create knock-out construct for the isolated gene. Transfect and isolate ES clones that have undergone gene targeting. Inject ES clones into blastocysts to generate a line of mice that harbor the gene knock-out mutation in their germline. Study phenotype of mice homozygous for the mutation. *Work on this task has not yet been initiated.*

## Conclusions

The main objective of the early stages of this grant is to gather reagents. Therefore, at this point in the grant, we are not able to draw any conclusions. Although we have encountered several problems, we continue to make progress and anticipate that we will have conclusions to report at this time next year.

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